A Quantitative Study of Destruction of Vitamin A in Butter Fat by Heating

By
KONRAD C. HSU

DISSERTATION

Submitted in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy
in the Faculty of Pure Science
in Columbia University

NEW YORK CITY
1924

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Dedicated

TO THE

MEMORY

OF THE LATE

KAN CHIU-NAM

1872-1923

A Pioneer of Modern Industry in China

ACKNOWLEDGMENTS

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A QUANTITATIVE STUDY OF DESTRUCTION OF VITAMIN A IN BUTTER FAT BY HEATING

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I. INTRODUCTION

The existence of a fat-soluble substance capable of inducing growth and protecting against nutritive failures and subsequent death, was first found in butter fat and egg fat by McCollum and Davis (1, 5) and Osborne and Mendel (2, 3) independently in 1913. This was demonstrated by the fact that an animal on a diet adequate in all respects except in this factor, would sooner or later decline in growth. At the same time the animal would be rendered more susceptible to various infections and die eventually if left long enough on this diet. Hence this factor is termed fat-soluble vitamin or growth-promoting factor. It has been found by various investigators later that this vitamin is quite extensively distributed in animal and plant tissues (4, 13, 22).

It was also first noticed by Osborne and Mendel (2, 3) that there is a relationship existing between this kind of nutritive failure and a kind of eye infection in rats. In animals suffering from a lack of this vitamin in their diet, this infection, known as ophthalmia, xerophthalmia, keratomalacia, etc., is often manifested at about the same time as the cessation of growth. This condition can be cured by re-introduction of the fat-soluble vitamin in the diet which simultaneously induces growth. It is thus also termed anti-ophthalmic vitamin (4). The term vitamin A without attachment of any particular property seems, however, the most common and unbiased term that can be applied to this factor.

The eye infection usually begins with a swelling of the eyelids and then develops an inflamed and catarrhal condition of the conjuctivæ with a bloody or purulent discharge (27, 37, 43). The sticky condition of the lids sometimes causes the eye to be completely closed. Sometimes the inflammation extends to the cornea. Yudkin and Lambert (28) reported that the first evidence of the eye changes consists of watery lacrimation with serosanguineous conjunctival secretion, becoming after a time somewhat viscid. These early focal lesions are found in the epithelial linings of the lids. The changes consist of localized foci of degeneration of the epidermis with cellular infiltration which, in some cases, extends into the sub-epidermal tissues. In all cases the cornea is not involved. This widespread keratitis is frequently complicated by corneal injury with infection and ulceration as a result. Mori found, however, that the xerosis is the essential change and it is caused by the dryness of the tissue. The ulcers of the cornea are secondary and are due to secondary infection by micro-organisms (27).

Daniels and Armstrong reported recently (30) that nasal sinusitis and general infection of the upper respiratory tract were noted in rats on diet deficient in fat-soluble vitamin. The nasal mucosa

was found to be covered with a thick creamy exudate which filled the meatus. The mid-ear cavity was filled with pus. Abscesses were also found at the base of the tongue. They concluded: "From the findings it would seem that fat-soluble vitamin plays an important role in the immunity of the organism to pyrogenic infection, and the general breakdown of the organism following the ingestion of diets low in fat soluble vitamin is secondary to such infections. A diet low in fat-soluble vitamin makes possible the bacterial invasion of the mucous membrane of the ear and nasal cavities."

Various workers have noted in animals deprived of vitamin A evidences of cutaneous malnutrition, such as, scabbiness of the tail and ear, sores or abnormal growth of the nose, thin and bushy hair and sore feet. Intestinal disorders as manifested by diarrhea with very penetrating odor were observed.

In short, the lack of vitamin A causes a general weakened condition of the body organism and thereby the animal is rendered susceptible to many kinds of infections.

There has been many an attempt to isolate and identify this vitamin, but none successful so far. Takahashi (29) reported that he had obtained a "semicrystalline active principle of 0.1% yield from cod liver oil, and 1 in 10,000 restores the life of an animal dying of lack of fat soluble vitamin." It is not a fat, since it stands drastic saponification (10, 25). It is not identical with glycerol, cholestrol, lecithin, sphingosin, phrenosin, cephalin, and lipochorome and carotin, and neither with the commoner fatty acids (10, 11, 15). It is not an ester (25). It is soluble in many organic solvents. Alcohol or ether or a mixture of both has been used generally for its extraction (10, 8, 21, 34, 36).

Most authors regard this vitamin as very sensitive to oxidation. As to the question of effects of heat, the evidence is conflicting. More definite data regarding the thermostability of the substance is highly desirable both because of its nutritional importance and as a means of advancing our knowledge of its chemical nature.

Because of individual variability among experimental animals an experiment on two to four rats can never be expected to yield a quantitative result even though the technique is accurate to the highest degree. To the writer it seems better to use all the animals that can be handled on one experiment and thus to work out really quantitative data. It is not uncommon to meet in the literature remarks upon "fallacy and danger of drawing conclusion from limited data" but apparently from 4 to 8 animals devoted to any one point have been considered sufficient in previous work upon the heat destruction of vitamin A. Study of the data here presented shows that with even 10 animals on any one point the average might be materially different from the average obtained from larger numbers (Tables V, VII, IX).

The present research is, therefore, planned on a strict quantitative basis with emphasis on fundamental principles rather than elaborate procedures. As many animals as could be handled were used in this investigation. The comparisons are made on several levels of heated and unheated materials, the object being to settle definitely the thermostability of vitamin A at 100 degrees Centigrade in butter fat as shown by heating experiments of 4 hours' duration.

II. PREVIOUS WORK

McCollum and Davis (6) saponified butter fat in a non-aqueous solution of alcoholic potash and extracted the active principle with olive oil from a solution of the resulting soaps. The conferring of growth-promoting activity on the olive oil led them to conclude that the vitamin is sufficiently stable to withstand the conditions of saponification employed. They also observed that the ether or petroleum ether extract of boiled eggs is active and therefore consider that this growth-promoting principle is resistant to heat (5).

In 1915 Osborne and Mendel (7) concluded from the results of experiments on growing rats that "heating butter fat with steam for two and one-half hours or longer did not destroy its growth-promoting efficiency." The two rats which they especially described received 18% of butter fat in the ration. The durations of the experimental periods were different. In another paper of the same year (7) they mentioned "no evidence of damage to the centrifuged milk by vigorous sterilization" (referring to vitamin A content).

Upon the stability of the growth-promoting substance in butter fat, Osborne and Mendel (11) reported that the butter fat stored for 15 months at 18° in light or in dark and at 8° in dark was still efficient like fresh butter. But in the case of butter "oil," in which this vitamin was more concentrated, gradual deterioration occurred, so that within a year this characteristic potency was eventually almost completely lost under the same conditions.

Steenbock, Boutwell and Kent (14) reported in 1918 that butter fat, when shaken in a glass stoppered bottle with distilled water the temperature of which was kept between 40° and 60° for 12 hours, was less efficient than unheated fat. The air in the bottle had been previously displaced by carbon dioxide. The rat described in each case gained 80 grams in four months. Butter fat through which had been passed a current of air at 100° for 12 hours did not promote growth or prevent death of the animal if 4% of this fat were included in the ration. Then three groups of rats, four in each, were placed on a diet deficient in vitamin A and supplemented

with heated and unheated butter fat. With 12% of fat heated for four hours at 100°, two animals gained 10 to 15 grams, one died and one just maintained its weight. While with 5% of unheated fat, animals gained from 60 to 80 grams. The experimental period was eight weeks. The heating was done in a pint mason jar at 100° in a Freas oven. They concluded "12% of butter fat heated for four hours at 100° was less efficient than 5% unheated fat." In other words, they considered that the heating of butter fat at 100° for four hours destroyed more than half of the growth-promoting vitamin in question.

In 1919 Drummond (17) investigated the effect of heat on vitamin A in butter fat by comparing the growth of young rats on unheated and heated butter fat as sole source of vitamin A in the diet. The experimental period used was five weeks. The typical animals receiving 10% of butter fat described in each case are as follows: On unheated fat the animal gained 71 grams. On fat heated at 100° for one hour the animal gained 3 grams (three weeks); on fat heated for two hours, 5 grams; on fat heated for four hours, 7 grams. On fat heated for four hours, at 75°, the animal gained 41 grams; on fat heated at 50°, 57 grams. The animal only gained 55 grams on the fat which had been kept for 21 days at 37°. Whereas the animal on 2% of unheated fat gained 29 grams. He concluded "vitamin A is destroyed by heating at 100° even for a duration of one hour," "some destruction" at 75° for four hours, "slight destruction" at 50° for four hours, and "very slight destruction" at 37° for 21 days. Further he added "Vitamin A is a substance of a much more unstable nature than had been previously believed." He also determined the iodine value of whale oil heated for four hours at 100° and obtained a difference of 2.3 in 118.5, but he did not regard this as an indication that the destruction was a result of oxidation.

Osborne and Mendel (24) reported in 1920 "butter fat heated in an air bath at 96° for 15 hours has not lost sufficient fat-soluble vitamin, if any, to make the product appear inferior to the original butter fat, when tested on rats that have declined from lack of fat-soluble vitamin."

Drummond and Coward (23) reported that the destruction of Vitamin A in butter fat was due to oxidation. This was shown by the fact that no destruction was indicated in the cases of fat heated at 100° for six hours, at 50° for six hours or at 96° for three hours. The heating was carried out in a current of live steam. But fat heated at 96° for 15 hours simultaneously with aeration gave results which indicated destruction of the vitamin. In the case of fat heated for six hours at 50° without aeration, two out of three rats described declined in growth, losing 5 and 10 grams respectively and the third gained 15 grams while with aerated fat treated in the same way, one lost 8, one gained 5 grams and the third just main-

tained its weight. They concluded "destruction of vitamin in butter fat occurs in presence of air. It is probably of an oxidative nature. Destruction takes place rapidly at high temperatures, but provided the exposure to air is extensive, considerable loss of nutritive value may take place at a temperature as low as 37°." In all these experiments butter fat was fed in separate doses of 0.2 gram daily. The duration of the experiments was five weeks in all cases.

Hopkins reported in 1920 (18) that butter fat aerated and heated at 120° for four hours in an oil bath when given to rats in amounts as high as 15 per cent of the ration as sole source of vitamin A did not protect the animals from keratomalacia. But butter fat heated at the same temperature for the same length of time in a steam autoclave when given to rats at the same level protected them from keratomalacia. He also obtained the following results: butter fat heated for 12 hours at 120° without aeration "vitamin A was somewhat destroyed, but far from being completely destroyed"; with aeration, at 120° for four hours, "destroyed greater part"; for 12 hours at 120° with aeration, "almostly completely destroyed"; for one hour at 120° with aeration, "less efficient for maintainence than unheated fat," and for two hours at 120° with aeration "increased destruction over that at one hour." The iodine value he obtained remained the same before and after heating even in the case of heating for four hours at 120° with aeration (39.0 and 39.0).

Steenbock, Sell and Buell (25) saponified the butter fat with alcoholic potash at 37° for four hours. Then the soap solution was extracted with ether three times. The ether extract was dried directly at room temperature in an air current. Under these drastic conditions the fat-soluble vitamin was not found to be destroyed to any appreciable extent.

III. METHOD

The methods of experimental work upon vitamins must be carefully considered if the results are to be made quantitative. Different laboratories employ different technique and as a result it is often impossible to correlate the data of different investigators. This is another of the great obstacles to progress along this line of investigation. A definite method must be standardized so as to overcome this difficulty.

Since there is no definite knowledge of the chemical nature of vitamin A the feeding methods must be used. For vitamin A work albino rats are generally used. They have various advantages over other animals. The short span of life, their moderate sensitiveness to vitamin A, and the smallness in size are the main reasons for their exclusive use in this work. Further their behaviors have been well standardized.

The various physiological effects produced by the vitamin A deficiency have been discussed. The manifestation of ophthalmia is not regular and time of onset varies (33). Osborne and Mendel observed 69 cases of ophthalmia out of 136 rats lacking vitamin A in the diet (40). Stephenson and Clark produced it in 28% of 46 rats (37). Walker secured, in one case, 5 in 64, and in another case 6 in 38 rats (38). Emmett reported the highest number, 120 in 122, or 98.3% of his rats lacking vitamin A in their diet contracted this infection (39). In the observations of the author most rats declining on a vitamin-A-free diet contracted this eye infection, but not all. Some rats may be of weakened appearance and losing weight and show the general appearance of vitamin A deficiency and yet not contract any eye inflammation, while some others of the same litter may develop very bad cases of ophthalmia. Further it is rather difficult to locate the time of the onset and the degree of the infection unless microscopic examinations of stained specimens are made. The determination of sinusitis also requires anatomical examinations which are not practical in the case of living animals. This leaves only the growth weight method as the possible indication of the general well being of the rats in a quantitative way. Zilva and Miura remarked "the only method is by growth. This although it may be vitiated by individual variations, has the advantage of regular manifestations. Rats which have ceased to grow owing to vitamin-A-deficient diet seldom do not respond to the addition of the active principle" (26).

Whenever biological methods are used the question of individual variation plays not an insignificant role. This can be eliminated to a certain extent by using more animals and restricting the comparison within the same litters, and lastly by employing larger numbers of animals of the same age. The question, how many animals should be used in each experiment, is rather difficult to answer. The laboratory work required necessitates some restriction in the size of the experimental groups (22). In this research 30 rats or more were used in each case where composite results were used for basis of comparisons leading to the conclusions. Probable errors are calculated in all cases so as to show as definitely as possible the degree of trustworthiness of the mean results.

Rats are known to be capable of storing considerable amounts of vitamin A in their bodies. Within limits this storage is proportional to the vitamin content of the previous diet. The young rats taken at weaning have stored considerable amounts of vitamin A obtained from the mother's milk. This is confirmed by various workers (41). Hence if the results are to be quantitative, this storage of vitamin of the rats must be standardized before the actual experiment commences. In this investigation young rats were taken at weaning and left on a vitamin-A-free diet until they ceased to grow. Then they were considered to be fit for the actual experimentation.

There is considerable confusion as to the exact amount of growth regarded by the different authors as "definite," "normal," or "efficient." Some workers prefer the normal growth curve as the indication of satisfactory growth and some regard a lower growth rate as more satisfactory. The minimum growth method seems to be the most quantitative of these known at present. The method as used by Sherman and Munsell (42) is to determine the minimum dosage of vitamin material used so as to give a very slightly upward curve for a period of eight weeks, and comparisons are made then on the dosages. This method has also been used by Sherman and Grose for vitamin B (46) and by Sherman, La Mer and Campbell for vitamin C determinations (48), and it proves to be most quantitative of all known methods.

IV. PROCEDURE

(1) *The Basal A-free Diet.*—The standard basal diet used should be free from vitamin A, at least containing no detectable amount, and at the same time should be adequate in all other respects. Only then can we correlate the animal behavior and this sole variable factor in the diet. Diet 280 of this laboratory has been used exclusively throughout the present experiments. It consists of:

Casein	12%
Flour ("patent")	78%
Salt Mixture (Osborne and Mendel)	4%
Dry Brewery Yeast	5%
Sodium Chloride	1%

The casein is purified by the method as used by Sherman and Munsell (42). Commercial casein is extracted three times with boiling 95% ethyl alcohol on a water bath under a reflux condenser for one hour each. For each extraction alcohol and casein are used in the proportion of 2:1 by weight. At the end of each extraction the casein is filtered while hot on a Buchner funnel with the aid of a suction pump. This procedure at least renders the vitamin A activity undetectable by the ordinary feeding method as used, while the addition of a small amount of butter fat alone makes the ration adequate for growth. Hence it can be regarded as vitamin-A-free.

Patent flour was used. It has been shown by various workers that such flour contains no appreciable amount of vitamin A. The salt mixture was prepared in the same way as described by Osborne and Mendel (15). Five per cent of dry brewery yeast in the ration, which corresponds roughly to 2.5 grams per animal per week (the food intake of the animal is about 50 grams per week) or 0.4 gram per day, is considered adequate by most authors as a source of vitamin B (12). Sodium chloride is added to make the food more palatable.

(2) *Vitamin A Material.*—It was in butter fat that vitamin A was first discovered. Most of the work recorded in the literature has been done on butter fat. Butter fat is one of the most common foods. It has a very high content of vitamin A. So butter fat was chosen as the vitamin A material in this investigation.

A sample of butter fat was specially prepared. The same butter fat was used throughout the entire research. It was prepared first by melting the butter at low temperature and then by being freed from the non-fatty ingredients by centrifuging. The containers were vacuum-sealed. This fat was first melted in a beaker at 40°. Then one portion was poured into a small weighing tube. The other portion was poured into a clean and dry specific gravity bottle. The bottle, filled with butter fat up to the top of the side tube, was immersed in a water bath. It was thus heated for four hours at 100°. Fifteen minutes were allowed for the fat to get heated up to the temperature. As the temperature rose the fat expanded and a small amount was forced out through the side tube. These droplets, squeezed out by the expansion of the fat, were carefully wiped off from time to time. After four hours the bottle was taken out of the bath. The portion of fat in the cap of the side tube was removed. Then the bottle with its contents was chilled and the fat poured into another weighing tube of the same size as that containing the unheated fat. The fat was then stirred until it solidified in order to insure uniformity of composition. These weighing tubes with the heated and unheated butter fat were stored in the refrigerator at 5 to 10° C. Fresh fat was prepared once every two weeks, or oftener if necessary.

Many workers had incorporated the vitamin material in their basal ration in different percentages. It can be easily seen that in such cases the exact amount of the vitamin material cannot be predetermined. Drummond and Coward (23) gave the vitamin material to the animals in separate doses daily. Sherman and Munsell (42) use about the same method except that in some cases the vitamin material was given three times a week instead of daily. The methods do not seem to be different materially, but the latter saves considerable labor. In this investigation portions of butter fat were weighed on a sensitive balance on watch glasses. The butter fat was then given to the animals on the same watch glasses. Weighings were made within 0.0005 grams. In all cases the animals licked up the fat immediately and completely.

(3) *Experimental Animals.*—Litters of healthy albino rats weighing between 28 and 60 grams were taken at the time of weaning, when 28 days old, placed in square cages (by the litter), and fed on the basal A-free diet *ad libitum* along with distilled water. The animals were weighed each week for the first four weeks. Later they were weighed once every other day, until they ceased to grow. This cessation of growth generally occurred between the

fifth and sixth week. Sometimes the earliest symptoms of sore eyes were manifested. The rats were then left at this constant weight stage for from two to four days, so as to be sure that they had definitely ceased to grow. Then the animals were transferred to individual cages and fed the same basal ration *ad libitum* and distilled water. In addition to this, varying doses of heated or unheated butter fat were given three times a week. Comparisons were made with rats of about the same size from the same litter. The animals were weighed each week and the amounts of food consumed recorded by weighing the collected residue and deducting it from the amount originally given. The mothers of the animals used had been raised on any one of a number of standard diets as used in this laboratory, chiefly mixtures of wheat and milk with or without additions of meat. The animals generally weighed between 60 and 100 grams at the time of separation (when growth had ceased on A-free diet), though the extreme upper and lower limits in weights of these animals were 126 and 42 grams respectively.

The experimental period was eight weeks in all cases. In cases of the negative controls and of the lower dosages of butter fat the animals were left on the experiment as long as they lived. The premature deaths were all recorded.

V. RESULTS

(1) *Negative Controls.*—The fact that land animals can store a certain amount of vitamin A in their livers and lesser amount in their adipose tissue fat has been demonstrated by various authors (31, 41). Sherman and Smith (43) state "That vitamin A may be stored to an important extent in the body is indicated not only by the results of feeding body fats . . . but also by the fact that when growing animals previously well fed are placed upon diets deficient in vitamin A and often apparently devoid of it, they usually continue to grow for some time, often doubling their body weight before signs of nutritive deficiency appear." The independent results of Goldblatt and Soames (32) and Coward, Lush and Palmer (36) obtained by feeding experiments with livers of rats which were previously depleted of their vitamin storage and also of those of normal animals confirmed the view that the livers of rats may vary greatly in their content of vitamin A.

Nevertheless very few of the previous workers laid any emphasis on this factor in vitamin A experiments. This vitamin A storage varies considerably with different animals, as shown by the results obtained by the author, as well as many other investigators in this laboratory. Thus the experiments conducted on animals which have

not been previously standardized in this respect, may yield misleading results.

In leaving the animals on the basal A-free diet described for two to four days after they ceased to grow so as to be sure of that the body store of vitamin A has been reduced to a minimum, care must be taken not to leave them too long. Many animals were sacrificed in the preliminary work just to locate the best point of commencing the actual experiments. After the animals had reached this stage, they were separated and placed on various levels of butter fat. Negative controls were taken from every litter and left on the basal ration until they died. These, besides demonstrating the absence of vitamin A in this diet, also served to show the survival period of the animals on such a ration. The following results were obtained from animals of all litters used in the experiment. Table I shows that the average survival of males varies from six to nine weeks, and that of females varies from six to eleven weeks, the average being 55.3 ± 2.0 and 58.3 ± 1.2 days respectively (see Chart I).

It is further indicated that the cessation of growth occurs in both sexes about the end of the fourth week and the rapid loss in weight does not occur until the sixth week. This suggests that the best time of commencing the experiment is between these two stages. Generally the animals were separated at the end of the fifth week, which is the midpoint of the almost horizontal portion of the curve. When animals were separated at this stage it was found that they responded to the addition of vitamin A material, even of very small quantity (shown by the tables III, IV). In other words these animals at this stage have their vitamin storage reduced to a minimum but are not permanently inhibited in growth as various authors have suggested.

TABLE I

NEGATIVE CONTROLS (ON BASAL DIET ONLY)
MALES

Rat	Previous	Initial	Gain by the week in grams									Survival
No.	Diet	Weight	1st	2nd	3rd	4th	5th	6th	7th	8th	9th	in days
10568	13	32	16	19	4	4	— 4	0	—15	— 6	..	61
10567	13	33	2	9	5	0	— 3	3	— 5	..	..	54
10548	13	49	20	26	17	10	—17	9	— 8	— 9	—10	68
10608	13	48	20	20	12	13	—13	14	—13	— 2	—21	67
11507	13	39	11	5	8	12	2	— 7	— 5	—10	..	56
11532	13	32	8	3	9	4	— 3	— 5	..	..	..	43
11714	13M	55	21	0	— 4	2	— 1	— 3	..	..	..	44
11715	13M	52	25	2	— 2	5	— 4	— 3	..	..	..	45
12721	13	30	12	1	3	0	— 4	— 2	..	..	..	43
12926	13	34	15	9	6	5	— 5	— 4	0	..	..	51
13068	13	39	14	19	5	5	4	0	— 5	— 3	— 1	67
13080	13	40	22	9	10	3	— 4	— 2	— 7	— 3	..	62
13105	501	30	16	12	11	6	4	— 3	— 5	— 1	..	62
Average			15.5	10.3	6.5	5.3	— 3.7	— 0.2	— 7.0	— 5.0	..	55.3 ± 2.0

TABLE II

NEGATIVE CONTROLS (ON BASAL DIET ONLY)

FEMALES

Rat No.	Previous Diet	Initial Weight	Gain by the week in grams 1st	2nd	3rd	4th	5th	6th	7th	8th	9th	10th	11th	Survival in days
9403	13	55	11	17	11	0	5	5	—14	8	—7	—7	..	72
10552	13	45	18	14	11	6	—1	—1	— 5	—2	—4	..	..	69
10593	13	40	19	11	8	0	3	—2	— 2	0	—6	..	..	67
10612	13	41	15	12	8	6	—7	7	— 1	—1	—4	..	..	66
10637	13	32	15	0	4	0	—1	2	— 3	—4	..	..	..	58
11516	13	34	9	5	11	—3	—2	—4	..	..	..	..	..	48
12735	13	32	22	14	9	—1	0	—5	— 4	..	..	..	..	56
12767	13	23	14	10	6	—3	—8	—2	..	..	..	..	..	48
12774	13	46	17	9	—5	—2	—1	—2	— 1	—1	—1	..	..	68
12795	13	54	8	9	5	—1	1	0	0	—1	—1	—2	—7	77
12811	13	23	9	14	8	4	3	—3	—10	—2	..	..	..	56
12893	13	39	9	8	0	2	1	0	— 3	..	..	..	..	54
12918	13	31	3	12	—4	—2	—2	..	..	..	..	..	..	40
12911	13	32	13	7	0	0	—3	—8	..	..	..	..	..	44
12933	13	38	3	15	10	7	—2	—1	0	—1	—6	..	..	63
12957	13	31	7	14	9	—4	—5	—4	..	..	..	..	..	42
12991	13	30	10	8	6	—1	—1	0	—2	—3	..	..	..	59
12998	501	30	16	9	0	—1	0	—4	1	—6	..	..	..	59
13038	502	37	17	18	6	12	—1	—2	—5	..	..	..	..	55
13057	13	35	15	9	0	5	1	5	—4	—3	—2	..	..	67
13090	13M	49	16	0	0	0	2	0	—2	0	..	..	..	62
13163	13M	51	4	3	1	12	—2	0	—1	..	..	..	..	54
13165	13	38	13	11	11	2	—1	—2	—4	—4	..	..	..	57
Average			12.3	10.0	5.0	1.7	—0.9	—0.9	—3.3	—1.5	—3.9	..	..	58.3±1.2

NEGATIVE CONTROLS

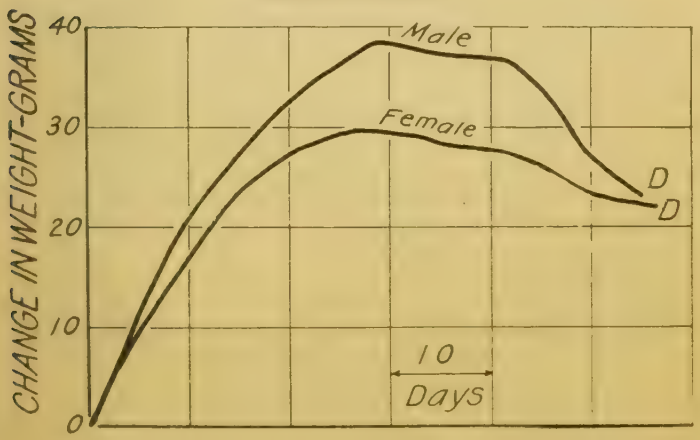

Chart I. Average gain curves of rats placed on diet 280 at 28 days of age.

(2) *Unheated Butter Fat.*—The last but not the least cause for the confusion concerning the heat destruction of vitamin A is the indefinite standard of comparison. Most authors compare the

results with that of the normal animals. Hopkins remarked (18) "Many authors seem content to compare growth obtained upon a given experimental dietary with the accepted 'curve of normal growth' . . . normal growth varies with season and stock." The most quantitative method of estimation of fat-soluble factor as noted by Zilva and Miura (26) and Sherman and Munsell (42) is the minimum growth method, i. e., by locating the smallest dose for maintenance or slight growth.

Animals of different sizes were first put on various levels of unheated butter fat. The results are shown in tables III, IV and V. Chart II shows that 12 or 13 mgs. of butter fat is about the minimum dosage for maintenance; 25 mgs. is much higher than the requirement for maintenance, although still giving less than the normal growth; 10 mgs. is just below maintenance. Twenty mgs. of the fat just gives a gain of 16 grams in eight weeks, which is certainly sufficient for good comparisons, as shown by the slow but steady upward trend of the curve, since the increase in weight at this level is more regular. Hence most animals were placed on this level and used as positive controls. A total of 37 animals were placed on this level.

The exact minimum dosage for maintenance is probably around 15 mgs. or rather between 20 and 13. This is shown by the fact that the loss and gain in the body weight of the animals produced by a decrease and increase of 5 mgs. of the vitamin material, are of the same order. But in the case of the lower dosage the animals are on the verge of physical break-down and hence are very susceptible to any pathological complication followed by immediate death which may be the result of secondary infection. This is indicated by the fact that 40% of the animals died before the end of the period. Even those which lived through the eight-week period were in very poor condition. On the other hand, those on 20 mgs. of butter fat were perfectly healthy.

TABLE III

WEEKLY GAINS IN WEIGHT OF RATS ON UNHEATED BUTTER FAT AS SOLE SOURCE OF VITAMIN A IN THE DIET

10 MGS DAILY EXCEPT SUNDAYS

Rat No.	Weight at Separation	Gain by the week in grams								
		1st	2nd	3rd	4th	5th	6th	7th	8th	Total
13082	91	—4	0	0	0	—1	—7	..	..	..
13166	81	—6	4	—3	—1	0	0	..	..	..
13160	110	—3	1	0	0	—2	—6	..	..	..
13103	103	0	—1	0	0	—1	—2	—7	—3	—14
13087	95	0	2	0	—2	—1	—4	—5	—3	—13
13089	88	0	—1	—5	0	0	—2	—4	—4	—16
13071	73	—5	4	0	—1	—3	0	0	—1	6
13055	90	0	—5	1	0	—3	—2	—1	—1	—11
Average		—2.2	0.5	—0.8	—0.5	—1.4	—2.8	—3.4	—2.4	—13.0

TABLE IV

WEEKLY GAINS IN WEIGHT OF RATS ON UNHEATED BUTTER FAT AS SOLE SOURCE OF VITAMIN A IN THE DIET

12 MGS DAILY EXCEPT SUNDAYS

Rat No.	Weight at Separation	Gain by the week in grams 1st	2nd	3rd	4th	5th	6th	7th	8th	Total
9407	84	5	1	3	0	0	—10	3	—2	0
9405	90	0	1	1	—1	—6	—4	0	—2	—11
Average		2.5	1.0	2.0	—0.5	—3.0	—7.0	1.5	—2.0	—5.5

13 MGS DAILY EXCEPT SUNDAYS

Rat No.	Weight at Separation	Gain by the week in grams 1st	2nd	3rd	4th	5th	6th	7th	8th	Total
9408	82	3	4	—3	—6	5	—4	5	1	5

25 MGS DAILY EXCEPT SUNDAYS

Rat No.	Weight at Separation	Gain by the week in Grams 1st	2nd	3rd	4th	5th	6th	7th	8th	Total
9370	85	0	7	6	1	3	7	1	—1	24
9372	75	3	4	6	3	2	11	—3	1	27
9373	64	5	2	3	3	4	1	0	9	27
Aevareg		2.7	4.3	5.0	2.3	3.0	6.3	—0.7	3.0	26

TABLE V

WEEKLY GAINS IN WEIGHT OF RATS ON UNHEATED BUTTER FAT AS SOLE SOURCE OF VITAMIN A IN THE DIET

20 MGS DAILY EXCEPT SUNDAYS

Rat No.	Weight at Separation	Gain by the week in grams 1st	2nd	3rd	4th	5th	6th	7th	8th	Total
10566	76	2	3	2	1	0	2	1	2	13
10547	98	2	0	2	7	0	2	1	1	15
10551	77	8	—1	5	2	1	2	1	2	20
10594	76	0	5	0	1	0	1	0	1	8
10606	102	6	—3	—5	4	4	4	3	2	15
10609	99	1	6	—1	6	0	—3	3	2	14
10635	61	1	2	0	2	0	2	0	3	10
11506	65	5	5	0	1	1	2	4	2	20
11512	59	2	2	7	0	2	3	3	2	21
11513	66	4	6	4	0	1	2	2	1	20
11535	50	1	3	1	2	1	3	3	4	18
11533	52	2	1	3	1	0	2	3	2	14
11722	72	1	3	1	1	2	5	2	0	15
11717	87	3	2	5	3	10	0	2	1	26
12723	50	0	1	3	5	3	0	1	1	14
12736	53	5	1	1	2	2	2	4	3	20
12764	70	—2	5	0	—1	1	2	2	2	9
12773	65	—4	5	6	4	2	1	6	—3	17
12792	109	1	0	2	2	2	0	1	1	9
12810	70	1	0	0	1	0	3	4	1	10
12892	77	2	1	2	3	2	2	3	2	17
[illegible]	[illegible]	4	3	[illegible]	[illegible]	5	1	1	[illegible]	21
12908	52	0	1	1	2	4	2	2	2	14
12932	71	0	1	1	2	—1	2	3	3	11
12928	75	3	2	2	0	2	1	2	3	15
12958	67	3	2	1	2	3	2	2	2	17
12990	63	5	2	3	1	1	2	—1	2	15
12995	69	5	3	3	2	3	2	—1	2	19
12996	70	5	3	5	4	1	3	—2	3	22
13036	104	2	2	1	3	2	3	1	1	15
13164	84	2	5	3	3	1	4	4	1	23
13159	118	2	1	4	1	3	3	2	2	18
13106	70	1	2	2	5	1	3	4	5	23
13084	116	2	4	3	2	0	1	1	5	18
[illegible]	93	3	2	1	0	1	3	1	2	11
13070	82	—2	2	1	2	1	2	1	6	13
13056	86	0	2	2	3	3	0	4	5	19
Average		2.0	2.3	2.0	2.2	1.8	1.9	1.9	2.1	16.2±0.17

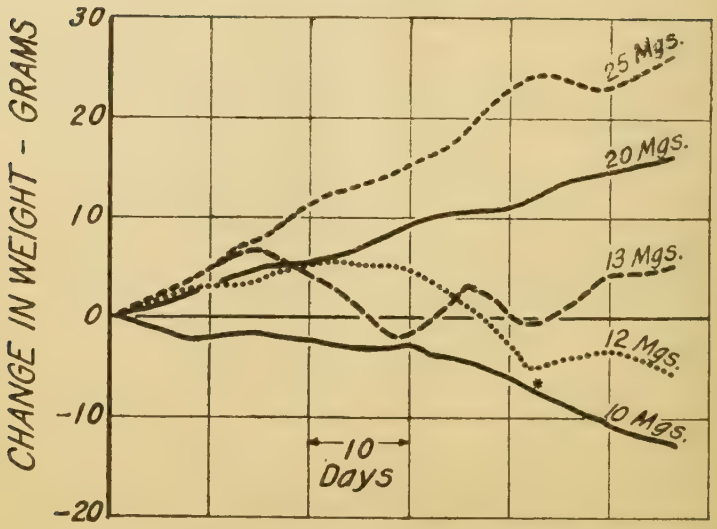

Chart II. Average gain curves of rats on varying doses of unheated butter fat as sole source of vitamin A in the diet after the animals ceased to grow on the A-free diet.

(3) *Heated Butter Fat.*—The effect of heating on butter fat at 100° for four hours was studied. Over 80 animals were employed on various dosages of heated butter fat. The preliminary results showed that 40 mgs. was approximately the amount required for slight growth. This indicated 50% destruction in the mode of heating described. Tables VI, VII, VIII, IX and Chart III show the results of the gains in weight of rats and their composite curves respectively.

The relation between the 15, 12.5 and 25 mgs. of heated butter fat was not very well shown, probably due to the fact that very few animals were used; but it does show that 15 and 12.5 mgs. are below maintenance, as none of the animals survived the experimental period, while 25 mgs. is around maintenance.

The rats on 30 mgs. of heated butter fat are at just about maintenance—possibly a little higher than the strict maintenance dose. Forty mgs. is decidedly more than enough for the maintenance requirement, while 20 mgs. is below maintenance. If there is an imaginary maintenance dosage which just produces a gain of 0 to 5 grams in weight it will be seen that the increase and decrease of 10 mgs. produce equal effect except in opposite directions.

* Indicates 40 per cent of animals died at this point.

TABLE VI

WEEKLY GAINS IN WEIGHT OF RATS ON BUTTER FAT HEATED AT 100° FOR 4 HOURS AS SOLE SOURCE OF VITAMIN A IN THE DIET

12.5 MGS DAILY EXCEPT SUNDAYS

Rat No.	Weight at Separation	Gain by the week in grams 1st	2nd	3rd	4th	5th	6th	7th	8th	Total
9369	85	0	1	1	0	—6	—9	..	..	
9402	89	5	1	0	—5	—1	..	..	..	
Average		2.5	1.0	0.5	—2.5	—3.5	..	..	..	

15 MGS DAILY EXCEPT SUNDAYS

Rat No.	Weight at Separation	Gain by the week in grams 1st	2nd	3rd	4th	5th	6th	7th	8th	Total
9371	77	6	8	—3	—1	..	..	..	..	
9404	85	2	0	—4	—3	..	..	..	..	
Average		4.0	4.0	—3.5	—2.0	..	..	..	..	

25 MGS DAILY EXCEPT SUNDAYS

Rat No.	Weight at Separation	Gain by the week in grams 1st	2nd	3rd	4th	5th	6th	7th	8th	Total
9374	73	3	4	1	0	1	1	—8	2	4

TABLE VII

WEEKLY GAINS IN WEIGHT OF RATS ON BUTTER FAT HEATED AT 100° FOR 4 HOURS AS SOLE SOURCE OF VITAMIN A IN THE DIET

20 MGS DAILY EXCEPT SUNDAYS

Rat No.	Weight at Separation	Gain by the week in grams 1st	2nd	3rd	4th	5th	6th	7th	8th	Total
9406	89	0	—7	—17	..	..	..	..	..	..
10565	81	—3	2	2	—5	0	0	—5	—2	—11
10553	77	2	3	2	2	—1	0	..	..	..
10605	94	—1	..	..	..	..	..	..	..	..
10607	103	1	—4	—2	2	—4	—2	0	—2	—11
10610	94	0	0	—4	0	0	—2	—1	—1	—8
10633	70	—4	0	—6	3	2	0	—5	..	..
11505	60	8	—2	—2	—2	..	..	..	..	..
11514	62	2	—2	0	—4	..	..	..	..	..
11537	52	4	0	—1	—2	..	..	..	..	..
11716	75	1	0	—6	..	..	..	..	..	..
11719	75	2	—1	—7	..	..	..	..	..	..
12722	59	—5	0	0	—4	..	..	..	..	..
12732	50	4	0	0	0	0	—1	..	..	..
12733	63	1	1	—1	0	—4	0	0	—2	—5
12763	71	—6	5	0	0	0	—2	—2	—1	—6
12791	126	0	—2	—6	0	0	—2	0	—3	—13
12794	86	0	0	1	—1	—1	0	—1	—4	—6
12913	83	—2	0	—1	0	0	0	..	..	..
12917	57	0	0	—1	0	—1	—1	—1	—3	—7
12929	68	—2	0	0	—3	—1	—2	0	—4	—12
12930	62	—1	0	—1	—5	—5	..	..	..	..
12959	60	1	—1	0	1	—1	..	..	..	..
12992	56	1	—1	0	—2	0	—1	—3	—4	—10
12997	73	2	—3	0	0	0	—1	0	—3	—5
13162	95	0	—2	0	0	0	—1	..	..	..
13104	93	0	1	0	—1	—3	—2	—3	—4	—12
13085	88	0	—7	—4	0	1	—4	—5	—2	—21
13088	95	—1	0	0	0	0	—4	—4	—6	—15
13069	65	—2	—1	5	—2	1	1	—1	—8	—7
Average		0.0	—0.7	—1.7	—0.8	—0.8	—1.2	—2.0	—3.2	—10.4±0.3

TABLE VIII

WEEKLY GAINS IN WEIGHT OF RATS ON BUTTER FAT HEATED AT 100° FOR 4 HOURS AS SOLE SOURCE OF VITAMIN A IN THE DIET

30 MGS DAILY EXCEPT SUNDAYS

Rat No.	Weight at Separation	Gain by the week in grams 1st	2nd	3rd	4th	5th	6th	7th	8th	Total
11510	54	0	—3	1	2	2	4	—1	—1	4
11515	61	4	5	4	—4	0	0	0	0	9
11536	51	1	1	0	—1	2	0	—1	—1	1
13085	100	[illegible]	1	1	1	..	..	..	..	..
13061	98	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	1	1	[illegible]	[illegible]
Average		1.4	1.0	1.2	—0.4	0.8	1.4	0	0.2	5.6

TABLE IX

WEEKLY GAINS IN WEIGHT OF RATS ON BUTTER FAT HEAT AT 100° FOR 4 HOURS AS SOLE SOURCE OF VITAMIN A IN THE DIET

40 MGS DAILY EXCEPT SUNDAYS

Rat No.	Weight at Separation	Gain by the week in grams 1st	2nd	3rd	4th	5th	6th	7th	8th	Total
10549	99	1	3	4	3	0	1	0	1	13
10554	80	2	4	6	3	0	1	0	1	17
10596	79	1	6	—6	3	0	1	1	2	8
10634	63	—1	3	—1	0	2	1	1	2	7
11509	52	3	—3	3	1	4	0	2	2	12
11511	60	10	5	0	0	3	0	2	2	22
11534	49	2	0	1	3	2	1	1	5	15
11720	67	4	1	2	3	2	3	1	0	16
11721	73	3	0	2	4	0	2	1	2	14
12725	46	0	3	0	4	1	2	1	1	12
12724	50	—1	3	4	4	1	1	0	1	13
12737	56	5	3	3	3	1	1	0	1	17
12734	58	6	2	2	1	6	1	2	1	21
12762	69	4	1	0	2	0	1	—1	0	7
12765	67	—2	8	2	2	2	1	2	—4	11
12775	65	1	3	2	2	2	1	4	2	17
12790	118	0	1	1	2	1	0	1	1	7
12793	100	1	1	2	2	2	0	2	2	12
12809	74	—4	0	1	0	1	3	4	4	9
12889	65	3	3	4	3	2	1	1	1	18
12891	79	3	6	2	2	1	0	—1	1	14
12914	67	3	2	2	2	2	2	4	4	21
12915	63	3	2	2	4	2	1	1	5	20
12907	60	5	3	3	1	1	1	2	2	18
12910	48	3	2	2	2	3	1	1	2	16
12931	71	1	0	1	1	2	2	2	2	11
12925	75	2	1	1	2	2	2	3	3	16
12927	71	1	2	2	3	1	3	1	2	15
12956	67	6	3	2	2	1	1	2	1	18
12955	69	3	2	2	—2	4	4	2	2	17
12989	63	5	5	1	—4	2	2	4	2	17
12993	76	4	2	2	2	4	2	3	3	22
12994	71	4	2	5	2	3	1	2	1	20
13035	112	4	4	2	1	0	1	0	2	14
13037	93	2	2	1	2	4	4	2	0	17
Average		2.5	2.4	1.7	1.8	1.8	1.4	1.5	1.7	15.0±0.2

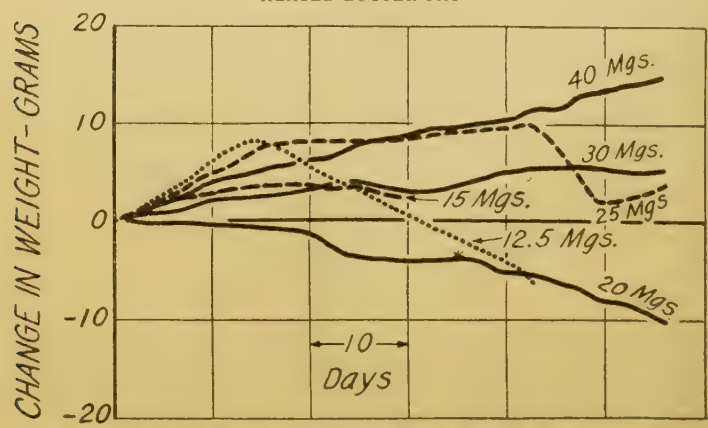

Chart III. Average gain curves of rats on varying doses of butter fat, heated at 100°C for 4 hours, as sole source of vitamin A in the diet after the animals ceased to grow on the A-free diet.

* Indicates 50% of animals died at this point.

(4) *Percent of Destruction.*—After having established the minimum dosages of unheated and heated butter fat for maintenance, animals were placed on two levels of unheated and three of heated fats. Negative controls were taken at the same time from every litter. The upper level of the fat intake is slightly above the maintenance requirement and the lower, below the maintenance. The object is to find besides the correlation in the corresponding doses of heated and unheated fat, if there is also any measurable relation between the growth curves resulting from different dosages. About 35 animals were used in each of the chief groups. If all these findings yield the same results within experimental errors, the interpretation of the effect of heat can be looked upon as conclusive and quantitative.

Tables III, V, VII, VIII and IX and the composite curves in Chart IV show the results obtained. The individual variation is quite large but owing to the great number of animals used the composite curve is quite smooth. The growth curves of animals on 20 mgs. unheated and 40 mgs. of heated butter fat almost coincide, those of the 10 mgs. unheated and 20 mgs. heated do not agree as well as the first pair of comparisons but are still of the same order. The error in the second pair of comparisons is greatly magnified by the fact that the animals were in a very poor nutritive state and hence were liable to any kind of pathological complication which depends greatly on the individual resistance. This is shown by the fact that the error of the mean result has increased to 8%. But the animals appeared to be in the same state of health in both cases, as shown by the fact that in such cases only 50% to 60% survived the eight-week period. The growth curve of the animals on 30 mgs. of heated butter fat, as shown in the chart, is about midway between the first two pairs of curves.

The curves of negative controls are plotted beginning from the time of separation, since the preliminary period is omitted in all cases. The curves indicate that the animals receiving even slight amounts of butter fat are much better off than those receiving none. Animals which received the lower dosages of the butter fat lived much longer in all cases than those on the basal diet alone. No negative controls lived more than 25 days after separation.

From the above results the only conclusion that can be drawn is that the vitamin A in the butter fat is destroyed to the extent of 50 per cent by the method of heating as described, i. e., at 100° Centigrade for four hours. The data as obtained and employed in these tables involve a minimum of error and the method of arriving at the per cent of destruction is the most simple and direct and probably most conclusive.

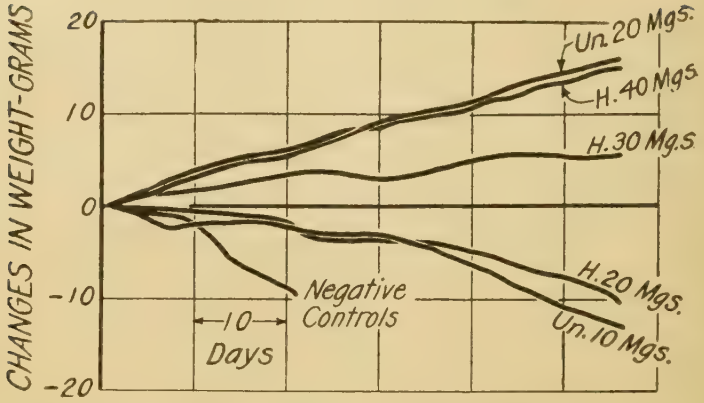

Chart IV. Comparisons of average gain curves of rats on varying doses of unheated and heated butter fat as sole source of vitamin A in the diet after the animals ceased to grow on the A-free diet.

VI. DISCUSSION

(1) *Errors Involved.*—Besides the question of animal variation, the most important known source of possible error is that of oxidation. There is probably a certain amount of oxygen dissolved in the butter fat in the process of its preparation. But during the heating there is certainly very little chance for any further introduction of atmospheric oxygen. Dyer (45) reported that on analyzing the air in the butter fat, nearly freed from non-fatty ingredients by melting, filtering and washing, and stored at —18°C. for three months, the oxygen was found to be present to the extent of 0.00012 mole per 100 grams of butter fat or 0.0038% by weight. The sample of butter fat used in this investigation probably contained less than that calculated by Dyer because the containers had been vacuum-sealed. No further attempt, however, has been made to free the butter fat used from the last trace of oxygen. The process of removing the last trace of air dissolved in the butter fat is rather a laborious task and would be of no practical importance for purifying the foodstuffs used for feeding experiments. Further in all previous work on vitamin A in butter fat, the materials used were not free from air. The long continued treatment of butter fat at temperatures above its melting point may further damage its vitamin content.

Hopkins (18) heated his butter fat in a current of live steam and found that the iodine value remained almost identical before

and after heating. For this purpose the author has also determined the iodine value by the method of Hanus (Table X). The average values obtained are 34.5 for the unheated fat and 34.0 for the heated which give a change of 0.5 in four hours of heating. But the probable errors of the values are ± 0.22 and ± 0.16 respectively. The precision of the method of Hanus probably does not exceed 0.25%. Hence it does not justify us to conclude that oxidation enters in to any appreciable extent in the method of heating. If there is any oxidation it cannot be large.

TABLE X

	Iodine Numbers						Average
Unheated Butter Fat.....	34.9	34.0	34.1	34.6	34.7	34.7	34.5±0.22
Heated Butter Fat.....	34.6	33.2	33.4	34.1	34.2	34.0	34.0±0.16
		Diff.	0.5±0.27				

Tests have been made as to the temperature of this mode of heating and these show that the variation of the temperature of the contents in the bottle is not more than 0.2° below the boiling water outside. The error of 0.2% is of a negligible magnitude in this type of experiment. The error in the time of heating cannot exceed five minutes in four hours, which correspond to an error of 1.6%. The dosage of butter fat was weighed within 0.5 mg. The rats licked up the butter fat almost as soon as it was placed in the cages. The probable loss of the fat in the process of eating does not exceed 0.5 mg. As there is no transferring involved in the feeding, the total error of the feeding cannot be more than 1 mg. This corresponds to an error of 2.5 to 5%. The results of the negative controls show that the storage of the vitamin in the body of the animal is well standardized before the experiment commences. There is still a very large possible error due to the factor of individual variation; but by using a large number of animals in each experiment it is considerably reduced. This can be seen by comparing the composite curves obtained from smaller number of animals and that of the larger numbers. The probable errors are calculated by the usual formula.* The values thus calculated show that the probable errors of the experiments vary from 2 to 8% of the magnitudes of the means. The average is around 5% which is probably a fair indication of the precision attained in the main results of this investigation.

Care has also been taken in storing both the heated and unheated butter fat in the containers of the same size. Both have been treated in the same way. So at any time the difference between the two samples of the fat is solely in the heating. The comparisons given above are made strictly within the same litter. Thus the probable error of the average figure cannot be more than ±5%.

(2) *Relation of Results to Previous Work.* The work of various authors has been described in detail in the first part of this

* As given, for example, by Jevons: Principles of Science, p. 337.

paper. Due to individual variation, the apparently small number of animals employed in different experiments did not warrant any quantitative conclusion. The animals used were generally not standardized as to their vitamin A storage. Hopkins (18) mentioned the advantage of this but used this method only occasionally. In 1921 Zilva and Miura (26) first used this for all experiments. "Animals weighing 50 to 60 grams are put on the basal diet. . . . Animals are kept three to four weeks thus on the basal diet before the actual experiment commences." But the previous diet of the animals was not given and the negative controls were not kept. Hence it is doubtful whether they have really obtained the stage of constant weight or beyond this stage. If the animals were kept too long on the A-free diet the minimum dose required is liable to be much higher than otherwise.

Most authors did not try to feed the vitamin A material aside from the basal ration, but incorporated the butter fat in the ration on different precentage levels. The daily intake of food of different animals varies not insignificantly. All these facts tend to increase the errors of the experiment.

As mentioned, several authors compared the results by using the "accepted normal growth curve" as the standard of comparison, rather than keeping positive and negative controls for each experiment. Chick and Hume (16) first noted the importance of the location of the minimum dosage of the unheated material before proceeding further in the experiment. It has been demonstrated by various authors that the best results obtained are on the dosages just slightly above maintenance. This difference in criteria used is one of the greatest causes of confusion in the figures obtained by different authors on the question of heat destruction.

Osborne and Mendel (7,24) and Hopkins (18) claimed that heating butter fat in steam does not affect the vitamin-A-content. They used 18% and 15% of butter fat in their rations. This corresponds to an intake of 8 to 9 grams per week which is about 80 times more than the actual requirement for maintenance. Sherman and Munsell (47) have secured growth of about the same order as that obtained by the author with 25 mgs. of butter per day as the sole source of vitamin A in the diet. This is in accord with the conclusion drawn here, that in most previous work the vitamin A material has been fed at too high levels for decisive quantitative determinations.

Drummond in 1919 (17) reported that the vitamin A in butter fat heated at 100° for one hour was totally destroyed. This was evidently due to the fact that the factor of oxidation was not avoided. But in the later work of Drummond and Coward (23) they found almost no destruction in heating butter fat in live steam at 100° for six hours. Their rats were not previously standardized. The dosage was 0.2 gram daily, which is 10 times the maintenance

requirement. These results are therefore also doubtful. Steenbock, Boutwell and Kent (14) obtained a result of more than 60% destruction by heating the butter fat in a mason jar in a Freas oven. They reported "5% raw fat is better than 12% heated fat in the ration." But seemingly too few animals were used.

In the present research all the controllable errors are either eliminated or reduced to a minimum and further the results are duplicated at different levels. This seems to leave very little doubt as to the exactness of the figures obtained.

VII: SUMMARY

(1) A quantitative method of determination of the effect of heating upon the vitamin A in butter fat with an error probably not exceeding $\pm 5\%$, is described; and reasons for previously discordant results are discussed.

(2) The storage of vitamin A in young white rats, four weeks of age, from mothers of the same diet, is studied by the weight curves and survival period on an A-free diet. The results show that the survival period of the males and females is not materially different. Both sexes cease to grow between the fourth and fifth week. The weight of the animals remained almost stationary for about a week and then dropped very rapidly. Hence the best time of beginning the experiment in the study of the vitamin-A-content of different foodstuffs is about the end of the fifth week. Further it is indicated that the survival has very little relationship with the initial weight of the animal within limits.

(3) Twenty mgs. of unheated butter fat per day given to rats which have been previously depleted of their vitamin A storage, along with a diet adequate in all other respects, is sufficient to maintain life and permit slight gain in weight. The exact minimum dosage for maintenance is probably between 13 and 20 mgs. daily.

(4) With a large number of rats the effect of heating on butter fat at 100 Centigrade for four hours is studied at different levels of vitamin A intake. All the results show definitely that the vitamin A in the butter fat thus treated is destroyed to the extent of 50 per cent.

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